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β-catenin is an important regulator of cell-cell adhesion and embryonic development that associates with and regulates the function of the LEF/TCF family of transcription factors. Mutations of β-catenin and the tumor suppressor gene APC occur in human cancers but it is not known if and by what mechanism increased β-catenin causes cellular transformation. This study was the first to show a serine phosphorylation-dependent regulation of β-catenin ubiquitination and degradation. We went on to demonstrate that modest over-expression of β-catenin in a normal epithelial cell results in cellular transformation. These cells form colonies in soft agar, survive in suspension, and continue to proliferate at high cell density and following γ-irradiation. Endogenous cytoplasmic β-catenin levels and signaling activity were also found to oscillate during the cell cycle. Taken together, these data point to a role of β-catenin in the regulation of the G_1 to S phase transition and suspension-induced apoptosis (anoikis). Additional results point to the important role played by two serine kinases (IKK and atypical PKC) in the normal phosphorylation and regulation of β-catenin signaling activity.

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Introduction

One of the hallmarks of the neoplastic process is the failure of transformed cells to stop dividing when in contact with their neighbors. The mechanism(s) whereby normal cells exhibit contact inhibition and the alterations in these pathways that result in the ability of cancer cells to overcome contact inhibition are poorly understood. Likely candidates for the transmission of the contact/proliferation signal are members of the cadherin family of cell-cell adhesion molecules, and their associated proteins, catenins.

β-catenin is also a member of the Wnt signaling pathway, which regulates several developmental pathways. Increases in cytoplasmic β-catenin and β-catenin signaling are also associated with numerous cancers. The oncogenic and developmental effects of β-catenin are mediated by its interaction with and activation of members of the LEF/TCF family of transcription factors. Although much is now known about this signaling system, the actual cellular processes in which β-catenin plays a regulatory role is unclear. As described above, it regulates cadherin-mediated cell-cell adhesion. Although it appears to regulate gene expression, few target genes have been demonstrated. Based on its relationship with Wnt and APC, it is possible that β-catenin may positively regulate cellular proliferation or inhibit apoptosis. It is also tempting to speculate that the adhesive and the putative oncogenic functions of β-catenin are related and that it may be, at least in part, the mechanistic link between cell-cell adhesion, contact inhibition and/or apoptosis. However, before our work, no studies directly tested the hypothesis that β-catenin is actually oncogenic.

Work carried out in this proposal demonstrate that β-catenin alters cell cycle progression and confers enhanced growth in soft agar, a surrogate marker for tumorigenicity. In addition, β-catenin confers resistance to suspension-mediated apoptosis (anoikis) and radiation damage and allows cells to continue cycling when cultured at confluence. In short, β-catenin transforms normal epithelial cells in culture.

The signaling activity of β-catenin is regulated at the level of cytoplasmic protein stability. The wnt signal transduction pathway regulates β-catenin stability by inhibiting the activity of the GSK-3/axin complex that, in the absence of wnt, is able to phosphorylate β-catenin and target it for ubiquitination. The N-terminal of β-catenin contains a region rich in serine and threonine residues that are likely targets for stability regulating kinases. Several of these potential phosphorylation sites are mutated in a number of different cancers strongly pointing to their importance in regulating the transforming activity of β-catenin. Only one of these serines is present within a GSK-3 consensus suggesting that other kinases are also likely to phosphorylate β-catenin and regulate its ubiquitination in a wnt and GSK-3-independent manner. Our results demonstrated that certain PKC inhibitors caused a dramatic accumulation of cytoplasmic β-catenin by inhibiting its ubiquitination. The inhibitor profile indicated that an atypical PKC regulates β-catenin accumulation. In addition, we defined a six amino acid motif that targets both β-catenin and the inhibitor of NFκB, called IκBα, for ubiquitination. A single serine to alanine mutation within this ubiquitination targeting sequence (UTS) stabilized the protein by inhibiting its ubiquitination. Further parallels between β-catenin and IκBα have since been discovered. The ubiquitin ligase complex that recognizes the IκBα and β-catenin UTS contains similar components. The identity of the kinase that directly phosphorylates the serines in the IκBα UTS was recently identified (IKK) and we show in our work that this complex is also involved in the regulation of β-catenin phosphorylation and signaling.

Methods and Results

The key methodology and results have been included in the annual reports and presented in 4 papers published in J Biol Chem and J Cell Biol. The full text of the papers is included in the appendix.

Key Research Accomplishments:

1. Demonstration that phosphorylation of β-catenin serine residues 33 and 37 targeted the protein for ubiquitination and subsequent degradation. This work formed the basis for many other studies from other laboratories that elucidated the nature of the SCF complex required for coupling phosphorylated β-catenin (and IκB) to the ubiquitination machinery.
2. The first demonstration that β-catenin was in fact oncogenic and regulated contact inhibition,anchorage-independent growth, anoikis and radiation-induced cell-cycle arrest.
3. The first demonstration that a kinase, other than GSK-3, was involved in β-catenin phosphorylation.
4. The first demonstration that significant cross-regulation of β-catenin and NFκB signaling pathways occurs through the IκB kinases.

Reportable Outcomes:

Lamberti, C., Lin, K-M., Yamamoto, Y., Verma, IM., Byers, S. and Gaynor, R. (2001) Regulation of β-catenin function by the IκB kinases J Biol Chem. 276:42276-42286.

Orford, K., Orford, C. and Byers, S. (1999) β-catenin regulates contact inhibition, anchorage-independent growth, anoikis and radiation-induced cell cycle arrest. J.Cell Biol. 146: 855-867

Easwaran,V. Song, V., Polakis, P. and Byers, S. (1999) The ubiquitin-proteosome pathway and serine kinase activity regulate APC modulation of β-catenin/LEF signaling. J. Biol Chem 274: 16641-16645.

Orford, K., Crockett, C., Weissman, A. and Byers, S.W. 1997. Serine phosphorylation-regulated ubiquitination and proteosomal degradation of β-catenin. J. Biol. Chem. 272: 24735-38

The three papers published before 2001 have already been cited over 100 times in the on-line literature. This translates to an impact factor of over 30/paper, not taking into account citations in reviews and other journals that are not on line. By any measure this is an outstanding citation index and indicates the general importance and impact of the work supported by this grant. This work was solely supported by the DOD for several years and emphasizes the important role of DOD funding

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Conclusions:

The wnt/β-catenin and NFκB pathways regulate the transcription of genes that are involved in cell cycle control and cellular differentiation. In addition the NF-κB pathway is involved in the induction of the inflammatory response. β-catenin, a known oncogene, is an important component of the wnt signaling pathway and IκBα is an important regulator of the NF-κB pathway. Both proteins are phosphorylated at

serines in the N-terminal region, which subsequently target them for ubiquitination by the same ubiquitin ligase complex. The kinases that are important in the phosphorylation of these proteins have been intensely studied. The IKK complex is responsible for the phosphorylation of IκBα while GSK-3β is thought to regulate β-catenin phosphorylation. The IKK complex contains two kinases, IKKα and IKKβ. Gene disruption studies in mice indicate that IKKβ is the dominant component of the IKK complex involved in phosphorylation of IκBα. We now show that IKK also exists in a complex with β-catenin and that expression of either IKKα or IKKβ can decrease β-catenin signaling in APC-mutant colon cancer cells with high endogenous β-catenin levels. However, only a dominant negative (DN) IKKα mutant increased β-catenin signaling and protein levels in cells with low endogenous β-catenin. In addition, DN IKKα, but not DN IKKβ, completely inhibited the ability of APC to decrease β-catenin signaling in colon cancer cells. These results indicate that, in contrast to IKK control of NFκB signaling, IKKα not IKKβ has the dominant role in the regulation of β-catenin activity.

Appendices (manuscripts and papers not previously submitted)

Lamberti, C., Lin, K-M., Yamamoto, Y., Verma, IM., Byers, S. and Gaynor, R. (2001) Regulation of β-catenin function by the IκB kinases J Biol Chem. 276:42276-42286.

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Regulation of β-Catenin Function by the IκB Kinases*

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Both the β-catenin and the nuclear factor κB (NF-κB) proteins are important regulators of gene expression and cellular proliferation. Two kinases, IKKα and IKKβ, are critical activators of the NF-κB pathway. Here we present evidence that these kinases are also important in the regulation of β-catenin function. IKKα- and IKKβ-deficient mouse embryo fibroblasts exhibited different patterns of β-catenin cellular localization. IKKβ decreases β-catenin-dependent transcriptional activation, while IKKα increases β-catenin-dependent transcriptional activity. IKKα and IKKβ interact with and phosphorylate β-catenin using both *in vitro* and *in vivo* assays. Our results suggest that differential interactions of β-catenin with IKKα and IKKβ may in part be responsible for regulating β-catenin protein levels and cellular localization and integrating signaling events between the NF-κB and Wingless pathways.

β-Catenin, the mammalian homologue of the *Drosophila armadillo* protein, is a ubiquitously expressed protein that has at least two distinct roles in the cell. First, it participates in cell-cell adhesion by mediating the association of E-cadherin with the cytoskeleton (1, 2). Second, it is a critical downstream component of the Wnt[1] or Wingless signal transduction pathway (3–5). The Wnt family of secretory glycoproteins plays an important role in embryonic development, in the induction of cell polarity, and in the determination of cell fate. Deregulation of Wnt signaling disrupts axis formation in embryos (5–8) and is associated with multiple human malignancies (9).

The current model of Wnt signaling indicates that the binding of the Wnt proteins to their receptor, frizzled, stabilizes β-catenin by inhibiting the activity of a serine/threonine kinase glycogen synthase kinase-3 or GSK-3β (9). GSK-3β is associated with β-catenin in a multiprotein complex that includes the adenomatous polyposis coli tumor suppressor protein (APC), axin or conductin, protein phosphatase 2A, and dishevelled. GSK-3β phosphorylation of residues in the amino terminus of β-catenin results in APC-mediated β-catenin degradation via the ubiquitin-proteosome pathway (10, 11). Increased levels of β-catenin are frequently found in colon cancer due to mutations in either the APC gene (12–14) or at residues in the amino terminus of β-catenin that are phosphorylated by GSK-3β (15–17). In the nucleus, β-catenin forms a complex with members of the T-cell factor (TCF)/lymphocyte-enhancer factor (LEF) family and activates gene expression of a variety of target genes (18–23) including c-*myc* (24) and cyclin D1 (25, 26).

NF-κB comprises a family of transcription factors which are critical in activating the expression of genes involved in the immune and inflammatory response and in the regulation of cellular apoptosis (27, 28). NF-κB is sequestered in the cytoplasm by a family of inhibitory proteins known as IκB. Upon stimulation of this pathway by a variety of agents including IL-1 and TNFα, the kinases IKKα and IKKβ (29–33) in conjunction with the scaffold protein IKKγ/NEMO (34–36) leads to the phosphorylation of IκBα at serine residues 32 and 36. Gene disruption studies in mice indicate that IKKβ appears to be the critical kinase in activating the NF-κB pathway (37–39), while IKKα appears to be critical for other functions such as keratinocyte differentiation (40–42). IKKα and IKKβ can form homodimers and also heterodimerize with each other, and this process is critical for their kinase activity. IKK phosphorylation of IκBα leads to its ubiquitination and degradation by the 26S proteasome and the nuclear translocation of NF-κB (43).

Interestingly, the sequence DSG*XX*S, which is the target site in IκB for IKK phosphorylation, is also found in the amino terminus of β-catenin (11). Phosphorylation of this sequence in both β-catenin and IκB stimulates their interactions with the ubiquitin ligase β-TrCP leading to their degradation by the proteasome (10, 11, 44). It has also been demonstrated that the β-catenin/TCF complex increases β-TrCP levels by a post-transcriptional mechanism to result in opposite effects on β-catenin and NF-κB activity (45). In addition, disruption of either the murine *GSK-3β* and *IKKβ* genes result in a similar phenotype with embryonic lethality due to hepatic apoptosis from increased sensitivity to TNFα (46). These results suggest potential relationships between β-catenin and NF-κB signaling pathways.

Given the fact that both the NF-κB and β-catenin pathways are important in the control of cellular proliferation and are regulated by cellular kinases that lead to β-TrCP-mediated degradation (10, 11, 45), we explored potential similarities in their regulation. First, we addressed whether there were differences in the cellular localization of β-catenin in wild-type mouse embryo fibroblasts as compared with fibroblasts derived from IKKα- and IKKβ-deficient mice. Next, we analyzed inter-

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[1] The abbreviations used are: Wnt, Wingless; APC, adenomatous polyposis coli tumor suppressor protein; TCF, T-cell factor; LEF, lymphocyte-enhancer factor; NFκB, nuclear factor κB; IKK, IκB kinase; MEF, mouse embryo fibroblast; FITC, fluorescein isothiocyanate; NIK, NF-κB inducing kinase; GST, glutathione *S*-transferase; PCR, polymerase chain reaction; HA, hemagglutinin; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.

actions between both IKKα and IKKβ and β-catenin and determined whether these kinases regulated β-catenin-dependent transcriptional activity. The results of this analysis indicate that IKKα can positively regulate β-catenin-dependent transcriptional activity while IKKβ negatively regulates this activity.

MATERIALS AND METHODS

Cells and Reagents—SW480 cells were purchased from American Tissue Culture Collection (Manassas, VA) and maintained in L-15 medium supplemented with 10% fetal bovine serum (HyClone Laboratories), 2 mM L-glutamine, and antibiotics (penicillin-streptomycin). COS, mouse embryo fibroblasts (MEFs, a kind gift of Xiaodong Wong), IKKα and IKKβ knock-out cells (39, 42) were maintained in Dulbecco's modified Eagle's medium and supplemented with the same components as above.

Antibodies—Polyclonal antibodies to IKKα (sc-7182), IKKβ (sc-7607), and β-catenin (sc-1496) were obtained from Santa Cruz Biotechnology. Monoclonal antibodies against β-catenin and TFIIB (Transduction Laboratory), IKKα (PharMingen), the hemagglutinin epitope HA/12CA5 (Roche Molecular Biochemicals), and the FLAG-epitope-M2 (Sigma) were also used in immunoprecipitation and Western blot analysis. Donkey anti-rabbit, anti-mouse and anti-goat antibodies conjugated with either anti-FITC or Red-X rhodamine were obtained from Jackson Laboratory.

Plasmid Constructs—The pCMV5 expression plasmids containing either FLAG-tagged IKKα and IKKβ including the constitutively active kinases (SS/EE) with substitutions at residues 176/180 for IKKα or 177/181 for IKKβ and the kinase defective (K/M) mutants at residue 44 in both IKKα and IKKβ were described previously (30, 47, 48). Wild-type and mutant IKKα and IKKβ cDNAs were each cloned into the baculovirus expression vector pAcHLT. The recombinant baculoviruses were used to infect SF9 cells to produce recombinant IKK proteins for *in vitro* kinase assays (48). The pCMV5 expression vectors containing the wild-type and the dominant negative NIK mutant in which lysine residues at positions 429 and 430 were substituted with alanine contained an amino-terminal Myc-tag (48).

The pCMV5 expression vectors encoding full-length human β-catenin was provided by S. Byers, while the plasmids for LEF-1, TOPFLASH, and FOPFLASH were gifts of K. Kinzler and R. Grosschedl. The RSV-β-galactosidase construct was a gift from P. Chaudhary. The glutathione *S*-transferase (GST) full-length β-catenin (GST β-cat-(1–781)) bacterial expression vector was constructed by using polymerase chain reaction (PCR) to generate a fragment encompassing the full-length β-catenin, which was then cloned in frame with GST in the pGEX. The GST fusion protein containing the amino-terminal 91 amino acids of β-catenin was constructed by *Sac*I digestion and ligation of the GST fusion containing wild-type β-catenin. The constructs GST-β-cat-(130–781), GST-β-cat-(1–400), GST-β-cat-(130–400), and GST-β-cat-(618–781) were constructed using PCR. The amino-terminal deleted form of β-catenin utilized PCR primers to generate a fragment containing amino acids 130–781, which was cloned into pCMV5 and contained a carboxyl-terminal HA-epitope. All constructs that were generated by PCR were subjected to DNA sequencing and cloned into pcDNA3.

Expression and Purification of GST β-Catenin Fusion Proteins—Recombinant GST β-catenin fusion proteins were expressed in bacterial strain BL21 and lysed in HMK buffer (50 mM Tris (pH 7.5), 0.1 M NaCl, 1 mM phenylmethylsulfonyl fluoride), and the bacterial lysates were incubated with 0.5 ml of packed glutathione-conjugated-agarose beads (Sigma) for 2 h at 4 °C. After three washes, the GST fusion proteins were eluted with 10 mM glutathione in HMK buffer and dialyzed, and protein purity was assessed by SDS-polyacrylamide gel electrophoresis.

Luciferase Reporter Assays—COS cells and mouse embryo fibroblasts were plated at 50% confluence in 35-mm tissue culture wells. After 24 h, the cells were transfected using LipofectAMINE Plus with the indicated DNA constructs and either the TOPFLASH luciferase reporter containing LEF/TCF binding sites or the FOPFLASH luciferase reporter with mutated LEF/TCF sites. An NF-κB luciferase reporter containing three NF-κB binding sites upstream of a thymidine kinase minimal promoter was used to detect NF-κB-directed gene expression. An RSV-β-galactosidase expression vector was included in the transfection assays to control for differences in transfection efficiency, and the pCMV5 plasmid was added to the transfection assays to standardize DNA quantities. Between 18 to 24 h posttransfection, the cells were washed twice with cold PBS, and the reporter activity was measured using the luciferase assay system (Promega). All transfections were done in duplicate and repeated at least three times.

Fractionation of Cellular Extracts—Cytoplasmic extracts were prepared from 10^8 SW480 or COS cells as described previously (49) with slight modifications. Cells were washed twice with cold PBS, and cell pellets were resuspended in 5 volumes of buffer A (10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM EDTA) supplemented with phosphatase inhibitors (10 mM NaF, 10 mM β-glycerophosphate, 0.5 μM okadaic acid, 1 mM sodium orthovanadate), and protease inhibitors (Roche Molecular Biochemicals). After incubation on ice for 10 min, cells were lysed with 15 strokes of a Wheaton all-glass Dounce homogenizer (Tight pestle). Nuclei were pelleted by centrifugation for 5 min at 2000 rpm (Beckman bench-top centrifuge, CH3.7 rotor). The supernatants termed S100 were collected, mixed with 0.11 volume of buffer B (0.3 M Hepes (pH 7.9), 30 mM $MgCl_2$, and 1.4 M NaCl), and then centrifuged at 100,000 × *g* for 60 min at 4 °C.

Whole cell extracts were prepared from COS cells transfected with hemagglutinin-tagged β-catenin alone or β-catenin and FLAG-tagged IKKα and IKKβ as described (47) in lysis buffer containing 40 mM Tris, (pH 8), 500 mM NaCl, 0.1% Nonidet P-40, 6 mM EDTA, 6 mM EGTA, 5 mM β-glycerophosphate, 5 mM NaF, 1 mM $NaVO_4$ (pH 10.0), and protease inhibitors (Roche Molecular Biochemicals).

Gel Filtration Chromatography—S100 extracts prepared from the SW480 and COS cells were further fractionated on a Superdex-200 gel filtration column (Amersham Pharmacia Biotech) and equilibrated with buffer D (20 mM Hepes (pH 7.9), 0.1 M KCl, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 0.2 mM EDTA, 20% glycerol). Protein markers (Sigma) used for the calibration of the column included bovine thyroglobulin (669 kDa), horse spleen apoferritin (443 kDa), β-amylase (200 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.5 kDa).

Protein Association and Western Blot Analysis—For endogenous protein association studies, equal volumes of proteins (200–300 μl) from each of the Superdex-200 fraction were incubated overnight at 4 °C with 1 μg of indicated antibodies or normal mouse IgG followed by the addition of protein G-agarose (Sigma) for 2–3 h at 4 °C. For protein association studies using transfected IKK and β-catenin expression vectors, COS cells were transfected with FLAG-tagged IKKα or IKKβ and HA-tagged β-catenin cDNAs. Cells were harvested 18–24 h after transfection, extracts were prepared, and gel chromatography was performed as described above. Equal volumes of each column fraction were immunoprecipitated with 12CA5 antibody or anti-FLAG M2 antibody. Immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech), and probed with specific antibodies. The membrane-bound immune complexes were analyzed with an enhanced chemiluminescence system (Amersham Pharmacia Biotech). For *in vitro* association studies, 40 μl of the cytoplasmic fractions were incubated overnight at 4 °C with 40 μl of the glutathione-conjugated-agarose bound with indicated proteins. Following three washes with 10 volumes of cold PBS, the protein complexes were subjected to Western blot analysis as described above.

In Vitro Kinase Assays—Kinase assays were performed as described by Yamamoto *et al.* (48). The baculovirus-produced IKK proteins were purified by nickel-agarose chromatography and then immunoprecipitated with 12CA5 monoclonal antibody (48). The epitope-tagged IKKα and IKKβ kinases were transfected into COS cells, and extracts were immunoprecipitated with the M2 monoclonal antibody directed against the FLAG-epitope. These kinases were added to kinase buffer containing 10 μCi of [γ-^{32}P]ATP, 1 mM ATP, 1 mM dithiothreitol, 5 mM $MgCl_2$, 100 mM NaCl, 50 mM Tris-HCl (pH 8.0), and then 1 μg of each of the substrates including wild-type or the S32A/S36A of GST-IκBα-(1–54) or GST-β-cat-(1–91) were incubated for 15 min at 30 °C. For determination of phosphate incorporation into the GST-IκBα-(1–54) and GST-β-catenin-(1–91), 2 μg of each of these substrates was incubated with either FLAG-tagged IKKα or IKKβ, which were immunoprecipitated from COS cell extract with the M2 monoclonal antibody in kinase buffer containing 15 μCi of [γ-^{32}P]ATP with a specific activity of 6000 Ci/mM (New England Nuclear) and either 0.01 mM, 0.01 mM, or 1.0 mM of cold ATP. The kinase reaction mixtures were subjected to electrophoresis on 10% SDS-polyacrylamide gels and autoradiography. The ^{32}P-labeled IκBα and β-catenin substrates were then subjected to scintillation counting, and the moles of phosphate incorporated were calculated. Reactions were incubated at 30 °C for 5, 15, 30, 60, and 120 min and stopped by the addition of protein loading buffer and heating to 90 °C.

Immunocytochemistry and Confocal Microscopy—Cells were cultured overnight on coverslips in Dulbecco's modified Eagle's medium without serum, washed two times with PBS, and fixed with 3.7% formaldehyde for 10 min followed by a brief permeabilization with 0.5% Triton X-100 in PBS. The cells were blocked for 30 min with 3% normal

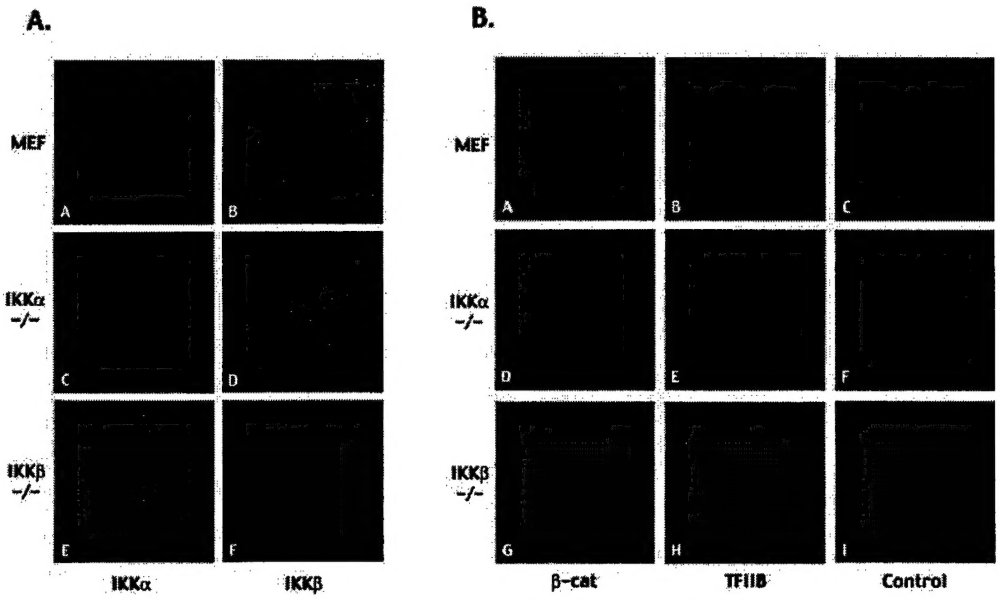

FIG. 1. **Characterization of β-catenin localization in IKKα- and IKKβ-deficient cells.** *A,* MEF (*panels A* and *B*) and either IKKα-deficient ($IKK\alpha^{-/-}$) (*panels C* and *D*) or IKKβ-deficient ($IKK\beta^{-/-}$) (*panels E* and *F*) embryo fibroblasts were plated overnight on coverslips before staining with either rabbit polyclonal antibodies directed against IKKα (*panels A, C,* and *E*) or IKKβ (*panels B, D,* and *F*) followed by staining with a secondary Red-X rhodamine-conjugated rabbit antibody. *B,* alternatively MEF (*panels A–C*), $IKK\alpha^{-/-}$ (*panels D–F*) and $IKK\beta^{-/-}$ (*panels G–I*) cells were stained with either a goat antibody to β-catenin (*green*) (*panels A, D,* and *G*) or a mouse monoclonal antibody to TFIIB (*red*) (*panels B, E,* and *H*). Donkey anti-goat antibody conjugated with FITC and anti-mouse conjugated with Red-X rhodamine were used as secondary antibodies. The respective negative controls utilizing the TFIIB monoclonal antibody and the donkey anti-goat FITC-conjugated antibody are also shown (*panels C, F,* and *I*). Images were collected using a laser scanning confocal microscope (Bio-Rad).

donkey serum in PBS and then incubated for 1 h with primary antibodies (diluted 1:50 to 1:200 in 1% normal donkey serum in PBS). The coverslips were washed three times with PBS and then incubated for 1 h with the secondary antibodies conjugated with FITC or Red-X rhodamine (diluted 1:400 in 1% normal donkey serum in PBS). Samples were washed three times and then treated with Aquamount (Polysciences). The results were analyzed on a laser scanning confocal microscope MRC 1000 (Bio-Rad).

RESULTS

β-Catenin Localization in IKK-deficient Cells—First, the localization of IKKα and IKKβ in wild-type MEFs was compared with that seen in IKKα-deficient ($IKK\alpha^{-/-}$) and IKKβ-deficient ($IKK\beta^{-/-}$) cells using immunofluorescence analysis with confocal microscopy. Wild-type mouse embryo fibroblasts (Fig. 1*A*, *panels A* and *B*), $IKK\alpha^{-/-}$ (Fig. 1*A*, *C* and *D*), and $IKK\beta^{-/-}$ (Fig. 1*A*, *panels E* and *F*) cells were plated on coverslips overnight and stained with rabbit polyclonal antibodies directed against either IKKα or IKKβ. In MEFs, IKKα localized in both the nucleus and the cytoplasm, while IKKβ localized predominantly in the cytoplasm (Fig. 1*A*, *panels A* and *B*). In $IKK\alpha^{-/-}$ cells, IKKβ localized predominantly in the cytoplasm (Fig. 1*A*, *panel D*). In $IKK\beta^{-/-}$ cells, there appeared to be increased IKKα present in the nucleus as compared with that seen in MEFs (Fig. 1*A*, *panel E*). There was no IKKα staining observed in $IKK\alpha^{-/-}$ cells (Fig. 1*A*, *panel C*) or IKKβ staining seen in $IKK\beta^{-/-}$ (Fig. 1*A*, *panel F*), thus confirming the identity of these cells.

Immunostaining of MEFs, IKKα-, and IKKβ-deficient cells with a polyclonal antibody directed against β-catenin demonstrated that β-catenin has a different pattern of staining in $IKK\alpha^{-/-}$ and $IKK\beta^{-/-}$ cells. β-Catenin was present in both the nucleus and the cytoplasm of MEFs with marked accumulation at cell-cell junctions (Fig. 1*B*, *panel A*). In $IKK\alpha^{-/-}$ cells, there was reduced nuclear staining of β-catenin as compared with MEF cells (Fig. 1*B*, *panel G*). There was more β-catenin present in the nucleus and the perinuclear region of $IKK\beta^{-/-}$ cells than in $IKK\alpha^{-/-}$ cells (Fig. 1*B*, *panel G*). As a control, these cells were also stained with a monoclonal antibody directed against the basal transcription factor TFIIB, which is localized predominantly in the nucleus (Fig. 1*B*, *panels B, E,* and *H*). There was little background staining when the FITC-conjugated anti-goat secondary antibody was used with the mouse monoclonal antibody directed against TFIIB (Fig. 1*B*, *panels C, F,* and *I*). These results indicate that there is less β-catenin localized in the nucleus of $IKK\alpha^{-/-}$ cells than in either $IKK\beta^{-/-}$ cells or MEF cells.

β-Catenin Activity in IKK-deficient Cells—Next we addressed whether the differences in β-catenin distribution in the IKK-deficient embryo fibroblasts could alter its transcriptional activity. The IKK-deficient cells and the parental MEFs were transfected with a TOPFLASH reporter construct alone or with expression vectors encoding either LEF-1 or β-catenin. The TOPFLASH reporter is driven by four LEF/TCF binding motifs inserted upstream of a minimal c-*fos* promoter and a luciferase gene (15). As a control, the FOPFLASH reporter, which lacks LEF/TCF binding sites, was utilized. An RSV-β-galactosidase expression vector was included in these transfections to control for differences in transfection efficiency.

When the TOPFLASH reporter alone was transfected into $IKK\alpha^{-/-}$ cells, there was consistently a 5–6-fold lower level of activity as compared with that observed in $IKK\beta^{-/-}$ cells (Fig. 2). Transfection of an expression vector encoding LEF-1 into either $IKK\alpha^{-/-}$ or $IKK\beta^{-/-}$ cells markedly stimulated TOPFLASH activity as did transfection of expression vectors encoding both LEF-1 and β-catenin. There was no significant activity from the FOPFLASH reporter in either the absence or presence of β-catenin and LEF-1 (Fig. 2). Transfection of expression vectors encoding wild-type IKKβ and LEF-1 into $IKK\alpha^{-/-}$ cells reduced TOPFLASH activity, while transfection of an IKKα expression vector with LEF-1 increased TOPFLASH activity in these cells (Fig. 2). When similar studies were performed in $IKK\beta^{-/-}$ cells, transfection of an IKKβ expression vector reduced TOPFLASH activity, while transfection of an IKKα ex-

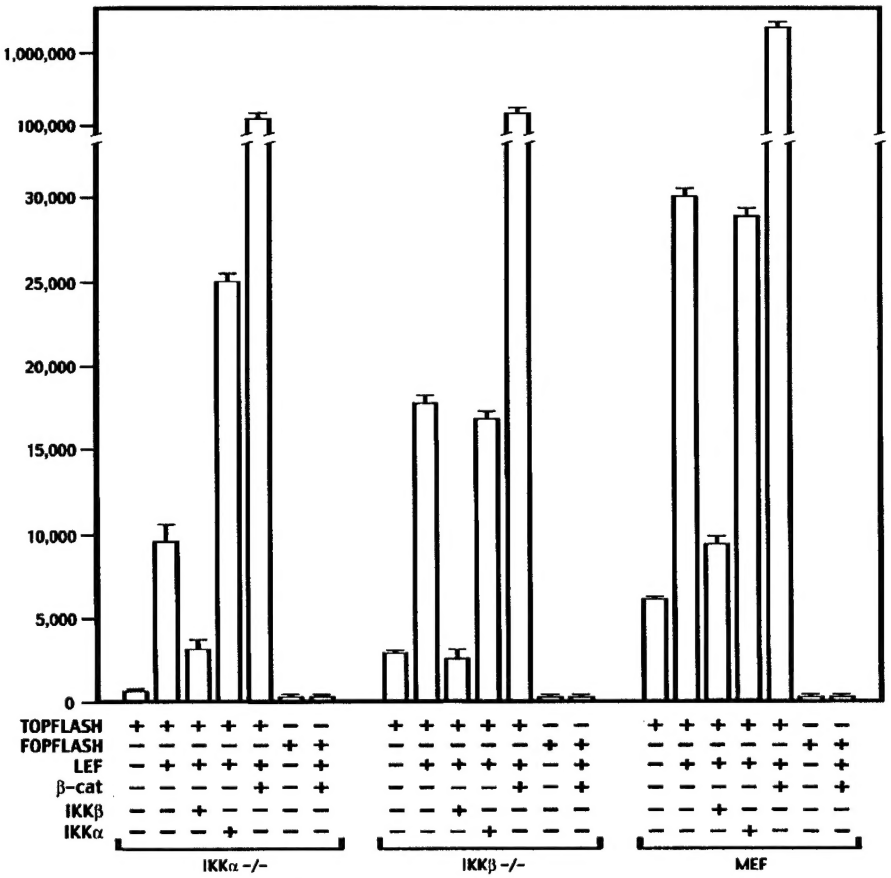

FIG. 2. **β-Catenin-mediated gene expression in IKKα- and IKKβ-deficient cells.** IKK$\alpha^{-/-}$, IKK$\beta^{-/-}$, and MEFs were each cotransfected with either a TOPFLASH or FOPFLASH reporter (0.85 μg) and pCMV5 expression vectors encoding β-catenin (0.5 μg), LEF-1 (50 ng), and either IKKα or IKKβ (0.5 μg) as indicated and an RSV-β-galactosidase reporter (0.60 μg) using LipofectAMINE Plus (Life Technologies, Inc.). All transfections contained 2.5 μg of DNA with a pCMV5 expression vector added to standardize DNA quantities. After 18 h, the cells were collected and lysed, both luciferase and β-galactosidase activity was determined, and the normalized luciferase activity was calculated by correcting for differences in β-galactosidase activity.

pression vector did not significantly alter TOPFLASH activity (Fig. 2). Transfection of both β-catenin and LEF-1 resulted in similar levels of TOPFLASH activity in the IKK$\beta^{-/-}$ and IKK$\alpha^{-/-}$ cells (Fig. 2). The parental MEF cells consistently gave somewhat higher TOPFLASH activity than seen in the IKK-deficient cells (Fig. 2). Again transfection of an IKKβ expression vector with LEF-1 into these cells reduced TOPFLASH activity, while transfection of an IKKα expression vector with LEF-1 resulted in little change in TOPFLASH activity (Fig. 2). Thus, the reduced levels of endogenous β-catenin in the nuclei of IKK$\alpha^{-/-}$ cells are associated with decreased β-catenin activation of gene expression, and this defect could be complemented by transfection of an IKKα expression vector. IKKα does not increase gene expression in the IKK$\beta^{-/-}$ and MEF cells, which have relatively abundant levels of nuclear β-catenin.

IKKβ and IKKα Have Differential Effects on β-Catenin Transactivation—The results presented in the previous section suggested that IKKβ and IKKα could potentially be involved in regulating the transcriptional stimulatory properties of β-catenin. Thus it was important to address whether either IKKα or IKKβ could alter β-catenin-mediated transcriptional activation in COS cells, which have low levels of endogenous β-catenin in the nucleus and relatively low levels of IKKα and IKKβ (data not shown). COS cells were transfected with either a TOPFLASH or FOPFLASH reporter, LEF-1 and β-catenin expression vectors, and increasing amounts of expression vectors encoding either the wild-type, constitutively active or kinase-defective mutants of IKKα and IKKβ. The constitutively active IKK proteins (IKKα SS/EE and IKKβ SS/EE) have glutamate substituted for serine residues in their T-loop so as to mimic phosphorylation of these residues and increase the activity of these kinases (30). The kinase-defective mutants (IKKα K/M and IKKβ K/M) contain a substitution of a lysine residue at position 44 with methionine (30).

As previously demonstrated, the coexpression of β-catenin and LEF-1 increased TOPFLASH but not FOPFLASH activity (Fig. 3*A*). When either wild-type IKKβ or the constitutively activate kinase, IKKβ SS/EE, was cotransfected with β-catenin and LEF-1, TOPFLASH activity decreased in a concentration-dependent manner (Fig. 3*A*). In contrast, cotransfection of either wild-type IKKα or the constitutively active kinase, IKKα SS/EE, increased β-catenin-dependent transactivation in a concentration-dependent manner (Fig. 3*A*). Transfection of the IKKβ K/M mutant resulted in a modest decrease in β-catenin transactivation that was not concentration-dependent, while transfection of the IKKα K/M mutant did not significantly alter β-catenin transactivation (Fig. 3*A*).

The cotransfection experiments in COS cells indicated that IKKα increased β-catenin-dependent gene expression, while IKKβ decreased β-catenin-dependent gene expression. Thus, we investigated whether IKKα and IKKβ can alter β-catenin protein levels. In addition, we asked whether IKKα and IKKβ

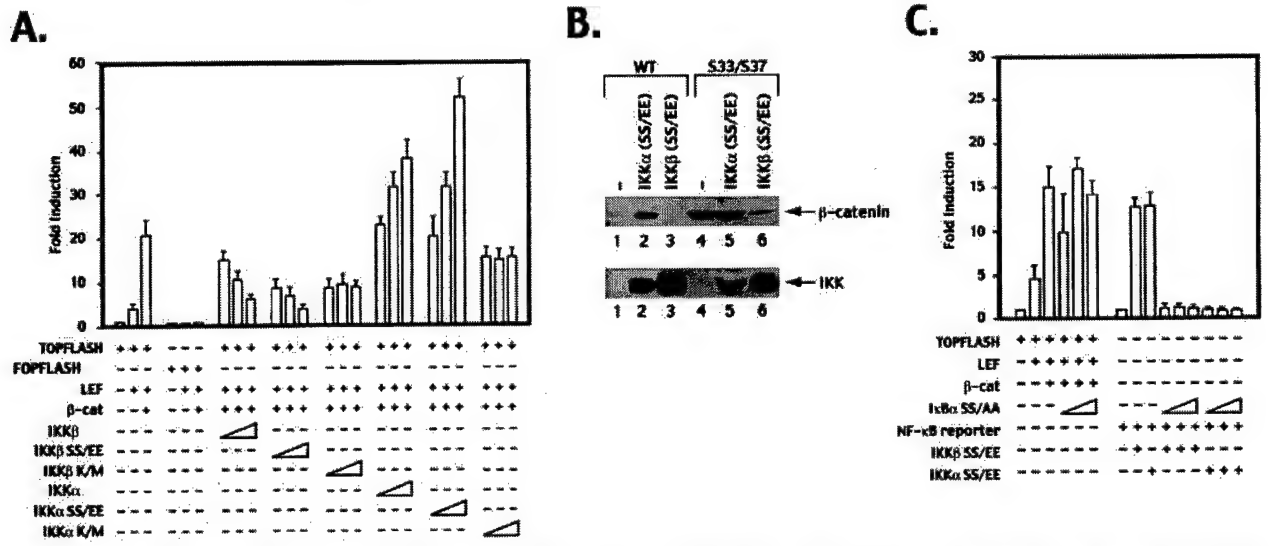

FIG. 3. **Role of IKKα and IKKβ in β-catenin transactivation.** *A*, the TOPFLASH reporter (0.75 μg) and expression vectors encoding LEF-1 (50 ng) and β-catenin (0.7 μg) were cotransfected into COS cells in the presence of wild-type IKKα or IKKβ, the constitutively active kinases, IKKβ SS/EE and IKKα SS/EE, or the kinase-defective mutants, IKKβ K/M and IKKα K/M, utilizing either 0.2, 0.5 or 1.0 μg of these kinases. *B*, COS cells were transfected with pCMV5 expression vectors encoding either HA-tagged wild-type (*lanes 1–3*) or the S33A/S37A mutant of β-catenin (0.7 μg) and pCMV5 (1.0 μg) (*lanes 1* and *3*) or either wild-type or mutant β-catenin together with an expression vector encoding the constitutively active FLAG-tagged IKKα (1.0 μg) (*lanes 2* and *4*) or FLAG-tagged IKKβ (1.0 μg) (*lanes 3* and *6*). Whole cell extracts were prepared and subjected to Western blot analysis with the anti-HA monoclonal antibody 12CA5 to detect the HA-tagged β-catenin (*lanes 1–6, top panel*) or the M2 monoclonal antibody directed against the FLAG-epitope to detect FLAG-tagged IKKα and IKKβ (*lanes 1–6, lower panel*). *C*, the TOPFLASH reporter was cotransfected with expression vectors encoding LEF-1 and β-catenin and the IκBα super-repressor (0.1, 0.2 and 0.5 μg) vectors. In addition, the NF-κB luciferase reporter (100 ng) was transfected with expression vectors encoding either the constitutively active IKKβ SS/EE or IKKα SS/EE and the IκBα super-repressor. All COS cell transfections contained 0.5 μg of an RSV-β-galactosidase expression vector, and the DNA amounts were standardized with a pCMV5 vector. Extracts were prepared 18 h after transfection, and the normalized luciferase activity was determined by correcting for differences in β-galactosidase activity. The change in gene expression relative to the TOPFLASH reporter alone was determined for each transfection and the average of three experiments (each in duplicate) is shown.

would affect the protein levels of a β-catenin mutant in which serine residues 33 and 37 were changed to alanine to result in increased β-catenin protein levels (15–17). Expression vectors encoding either the hemagglutinin-tagged wild-type or S33A/S37A mutant β-catenin were transfected into COS cells either alone or in the presence of either the constitutively active FLAG-tagged IKKα or IKKβ. Whole cell extracts were prepared from the transfected COS cells and analyzed by Western blot analysis using the 12CA5 and M2 monoclonal antibodies directed against the hemagglutinin and FLAG epitopes, respectively (Fig. 3*B*). IKKα expression increased the level of the epitope-tagged β-catenin protein (Fig. 3*B*, *lane 2*), while IKKβ reduced the amount of the epitope-tagged β-catenin protein (Fig. 3*B*, *lane 3*). In contrast, IKKα did not alter the level of the S33A/S37A β-catenin mutant (Fig. 3*B*, *lane 5*), while IKKβ reduced the level of this protein (Fig. 3*B*, *lane 6*). Transfection assays with the TOPFLASH reporter indicated that IKKα increased gene expression in the presence of the wild-type but not the mutant β-catenin, while IKKβ reduced gene expression in the presence of both of these β-catenin proteins (data not shown). These results suggest that IKKα either directly or indirectly may lead to increased levels of β-catenin to increase TOPFLASH activity, while IKKβ may reduce the levels of β-catenin to decrease β-catenin activity. The failure of IKKα to further increase the protein levels of the mutant β-catenin suggests that the structure of the amino terminus of β-catenin may be important in this process.

It was important to determine whether activation of the NF-κB pathway may be involved in the increased TOPFLASH activity seen in the presence of β-catenin and LEF-1. The TOPFLASH reporter was transfected with expression vectors encoding β-catenin, LEF-1, and the IκBα super-repressor (IκBα SS/AA) (Fig. 3*C*). The IκBα super-repressor protein, which contains substitutions of serine residues 32 and 36 with alanine, cannot be phosphorylated by IKK, and its resistance to degradation prevents the nuclear translocation of the NF-κB proteins in response to activators of this pathway (27). The transfection of the IκBα super-repressor did not alter activation of the TOPFLASH reporter in the presence of β-catenin and LEF-1 expression vectors, while it completely abolished the activity of an NF-κB reporter (Fig. 3*C*). These results suggest that NF-κB activation does not appear to be involved in the activation of TOPFLASH activity by β-catenin and LEF-1.

The Amino Terminus of β-Catenin Is Critical for IKKα but Not IKKβ Modulation of Gene Expression—Next we addressed whether the same or different domains in β-catenin were required for regulation by IKKα and IKKβ. The amino terminus of β-catenin is phosphorylated by GSK-3β leading to β-catenin degradation (50–52). Amino-terminal deletion mutants of β-catenin are very stable because they lack sequences that are involved in APC-mediated degradation (50–54). Furthermore, our results suggested that the amino terminus of β-catenin may be involved in IKKα-mediated regulation. To determine whether the amino terminus of β-catenin was critical for mediating the effects of IKKα and IKKβ, transfection of increasing amounts of a β-catenin expression vector deleted of its first 129 amino acids was transfected into COS cells along with LEF-1. There was increased TOPFLASH activity seen with this mutant similar to the results seen with wild-type β-catenin (Fig. 4). The expression of the constitutively active IKKβ protein reduced activation of TOPFLASH reporter when transfected with this β-catenin mutant. In contrast, the expression of the constitutively active IKKα protein did not alter the ability of the amino-terminal deletion of β-catenin to activate the TOPFLASH reporter (Fig. 4). These results suggest that the decreased β-catenin transactivation observed with IKKβ is not dependent on the amino terminus of β-catenin, while IKKα requires the presence of this domain to stimulate β-catenin transcriptional activity.

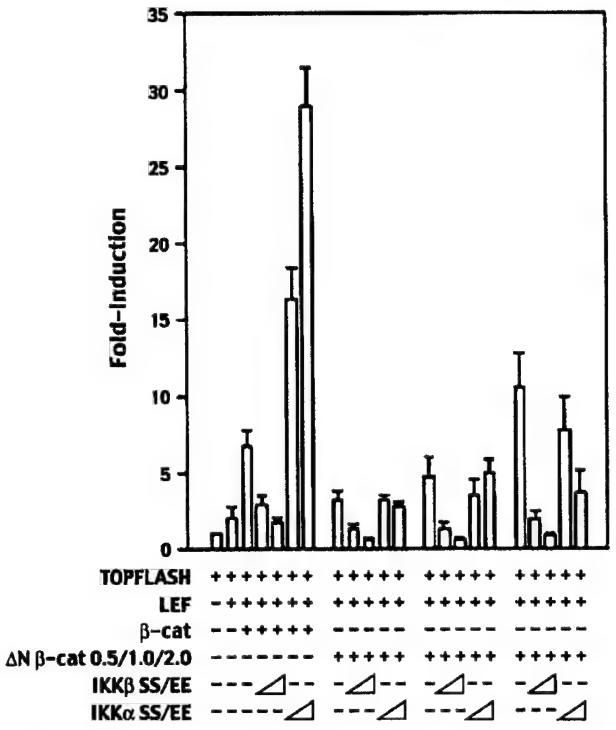

FIG. 4. **Differential effects of IKKβ and IKKα on transactivation of an amino-terminal truncated β-catenin.** COS cells were cotransfected with the indicated plasmids including the TOPFLASH reporter, LEF-1, and either wild-type β-catenin (*group 1*) or an amino-terminal deletion of the first 129 amino acids of β-catenin (*groups 2–4*). The construct encoding the amino-terminal-deleted β-catenin was cotransfected at concentrations of 0.5 (*group 2*), 1.0 (*group 3*), and 2.0 μg (*group 4*) together with the constitutively active kinases IKKβ SS/EE (0.5 and 1.0 μg) and IKKα SS/EE (0.5 and 1.0 μg). An RSV-β-galactosidase expression vector was added to each transfection, and DNA quantities were standardized by addition of a pCMV5 expression vector. After 18 h, the cells were collected and luciferase activity was determined and normalized to correct for differences in β-galactosidase activity. The change in gene expression relative to the TOPFLASH reporter alone was determined for each transfection and the average of three experiments (each in duplicate) is presented.

β-Catenin Interacts with IKKα and IKKβ—To address whether the effects of IKKα and IKKβ on β-catenin-dependent gene expression may be mediated by direct interactions with β-catenin, we performed coimmunoprecipitation experiments of β-catenin and the IKK proteins using cytoplasmic extracts prepared from the SW480 colon cancer cell line. SW480 cells express a truncated APC gene product and result in enhanced levels of β-catenin. This increased level of β-catenin was necessary to detect this protein in Western blot analysis of column fractions that were generated following chromatography (14).

Superdex-200 gel filtration chromatography of the S100 cytoplasmic extract prepared from SW480 cells was utilized to assay interactions between the IKK and β-catenin. Similar chromatographic analysis has previously been used to characterize the high molecular weight IKK complex (55). Western blot analysis of these column fractions indicated that β-catenin was present in a broad peak, including a portion that was present in high molecular weight fractions that also contained IKKα and IKKβ (Fig. 5*A*, *left panel*). Column fractions 7–12, which contained both β-catenin and the IKK proteins, were immunoprecipitated with a monoclonal antibody directed against β-catenin followed by Western blot analysis with either β-catenin, IKKα, or IKKβ antibodies (Fig. 5*A*, *middle panel*). This analysis indicated that β-catenin was associated with IKKα and IKKβ, while immunoprecipitation of these column fractions with mouse IgG followed by Western blot analysis demonstrated no association of these proteins (Fig. 5*A*, *right panel*). These results suggest that endogenous β-catenin can associate with IKKα and IKKβ.

Next, we characterized the interactions of IKKα and IKKβ with β-catenin following cotransfection of COS cells with expression vectors encoding these epitope-tagged proteins (Fig. 5*B*). First, S100 extracts prepared from these cells were subjected to Superdex-200 chromatography and Western blot analysis. As previously noted, when IKKα and IKKβ were transfected into COS cells they migrate in a relatively broad peak following Superdex-200 chromatography due to the failure to completely assemble into the high molecular weight IKK complex (49) (Fig. 5*B*, *left* and *middle panels*). Next, immunoprecipitation of column fractions prepared from extracts containing the FLAG-tagged IKKα and HA-tagged β-catenin was performed followed by Western blotting. This analysis indicated that FLAG-tagged IKKα and HA-tagged β-catenin were able to associate (Fig. 5*B*, *left panel*). Column fractions of extracts prepared from COS cells cotransfected with FLAG-tagged IKKβ and HA-tagged β-catenin indicated that both of these proteins were also able to associate (Fig. 5*B*, *middle panel*). Western blot analysis of the immunoprecipitated IKK and β-catenin proteins do not strictly overlap. This is likely due to the fact that their elution profiles following chromatography vary, which is consistent with the presence of these proteins in multiple complexes. The column fractions containing HA-tagged β-catenin and either FLAG-tagged IKKα or IKKβ were also immunoprecipitated with mouse IgG and analyzed by Western blot analysis. This analysis revealed that there were not nonspecific associations of the β-catenin and IKK proteins (Fig. 5*B*, *right panel*).

To further characterize the interactions of β-catenin with IKKα and IKKβ, *in vitro* binding of SW480 cytoplasmic extract with GST proteins fused to different domains of β-catenin was performed. Thus, we could determine the role of different domains of β-catenin including the amino terminus, which regulates protein stability, the armadillo repeats, and the C-terminal transactivation domain in binding the IKK proteins (53). Following the incubation of the SW480 cytoplasmic extract with the GST-β-catenin fusion proteins bound to glutathione-Sepharose beads, Western analysis was performed with antibodies directed against either IKKα or IKKβ. Each of the β-catenin fusion proteins, but not GST alone, was able to interact with IKKα and IKKβ (Fig. 6*B*). However, the GST-β-catenin fusion proteins extending between amino acid residues 1–400 and 130–400 consistently bound more IKKα and IKKβ (Fig. 6*B*). These results suggested that the region of β-catenin containing the first six armadillo repeats was probably critical for interaction with the IKK proteins. The data from the GST-pull down assays in conjunction with coimmunoprecipitation data of both endogenous and transfected proteins demonstrate that the IKK proteins and β-catenin can interact under a variety of different conditions.

IKKα and IKKβ Phosphorylate β-Catenin—Next we addressed whether IKK could phosphorylate the amino terminus of β-catenin and whether stimulation of IKK activity could result in increased β-catenin phosphorylation in *in vitro* kinase assays. The amino terminus of β-catenin has been demonstrated to be a target for GSK-3β phosphorylation (9), while serine residues 32 and 36 in the amino terminus of GST-IκBα are the target for IKK phosphorylation (29–33). HeLa cells were either untreated, treated with TNFα, or transfected with an expression vector encoding NIK (56, 57) to induce IKK kinase activity. The IKK complex was immunoprecipitated from extracts prepared from these cells and assayed for its

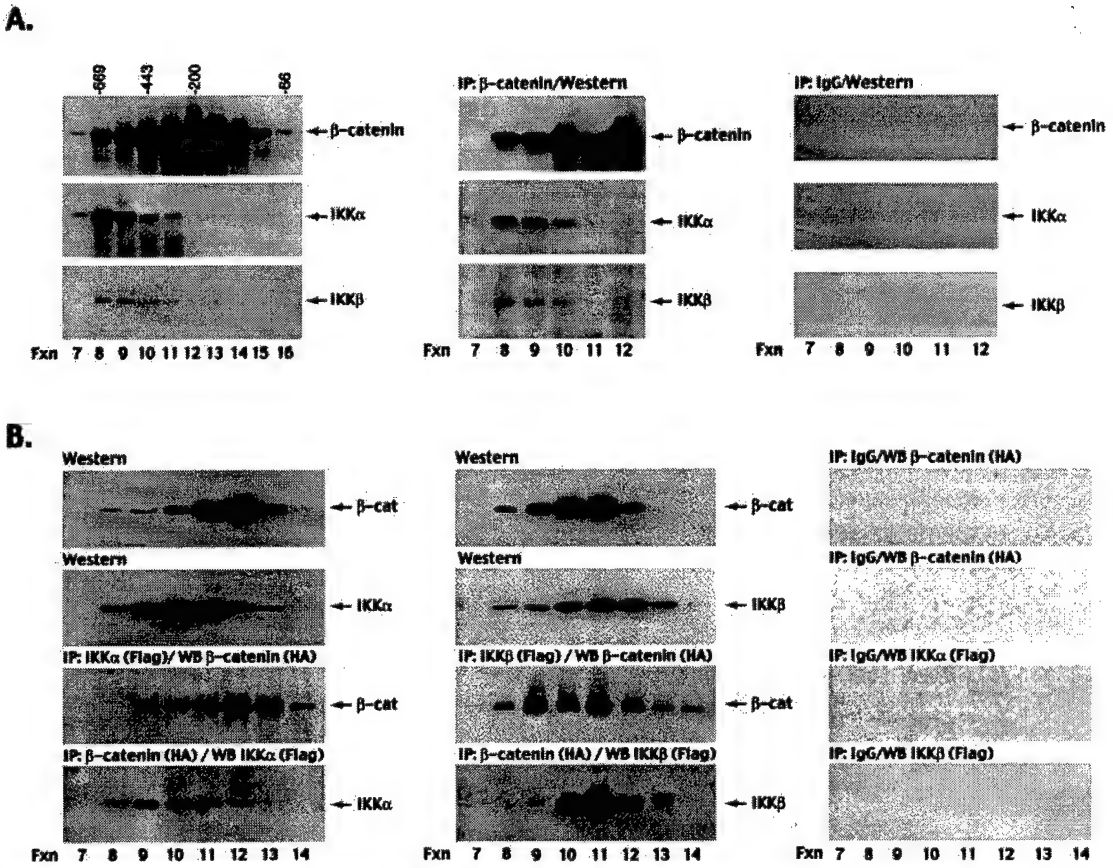

FIG. 5. **Association of endogenous and transfected IKKα, IKKβ and β-catenin proteins.** *A*, S100 extract was prepared from 10^8 SW480 cells and fractionated on a Superdex-200 gel filtration column. Equal volumes (40 μl) from each fraction were immunoblotted to monitor distribution of β-catenin, IKKα, and IKKβ (*left panel*). The mobility of the different protein markers on the Superdex-200 column are indicated as are the column fraction numbers. For association studies, equal volumes (200 μl) from *column fractions 7–12*, which contained the β-catenin and the IKK proteins, were incubated overnight with monoclonal antibodies directed against β-catenin (Transduction Laboratories). Western blot analysis was then performed on these immunoprecipitates using polyclonal goat antibody to detect β-catenin or rabbit polyclonal antibodies to detect IKKα and IKKβ (*middle panel*). These column fractions were also immunoprecipitated with mouse IgG followed by Western blot analysis with polyclonal antibodies directed against either β-catenin, IKKα, or IKKβ (*right panel*). *B*, COS cells were transfected using LipofectAMINE Plus with expression vectors encoding HA-tagged β-catenin and either FLAG-tagged IKKα or FLAG-tagged IKKβ. Cells were harvested 18 h after transfection, and S100 extracts were prepared and fractionated on a Superdex-200 column. Equal column volumes (40 μl) were subjected to Western blot analysis using 12CA5 antibody to detect HA-tagged β-catenin (*left* and *middle panels*) or the M2 monoclonal antibody to detect FLAG-tagged IKKα (*left panel*) or FLAG-tagged IKKβ (*middle panel*). Immunoprecipitation of column fractions prepared from FLAG-tagged IKKα and HA-tagged β-catenin (*left panel*) or FLAG-tagged IKKβ and HA-tagged β-catenin (*middle panel*) transfected cells was performed using either the 12CA5 or M2 monoclonal antibodies followed by Western blot analysis with the antibody that was not used in the immunoprecipitation. Column fractions from extracts containing either FLAG-tagged IKKα and HA-tagged β-catenin (*right panel*, *first* and *third gels*) or FLAG-tagged IKKβ and HA-tagged β-catenin (*right panel*, *second* and *fourth gels*) were immnoprecipitated with mouse IgG followed by Western blot analysis with either 12CA5 (*HA*) or M2 (*FLAG*) antibodies as indicated.

ability to phosphorylate either GST-β-cat-(1–91), GST-IκBα-(1–54), or GST-IκBα (SS/AA)-(1–54). IKK activity was induced by treatment with either TNFα or NIK and increased the phosphorylation of β-catenin (Fig. 7*A*, *lanes 1–3*) and IκBα (Fig. 7*A*, *lanes 4–6*), but not the IκBα mutant in which serine residues 32 and 36 were changed to alanine (Fig. 7*A*, *lanes 7–9*).

Recombinant baculovirus-produced IKKα and IKKβ were also tested in *in vitro* kinase assays using GST fusions with β-catenin or IκBα. Both IKKα and IKKβ also phosphorylated the amino terminus of β-catenin and IκBα, but not the IκBα mutant (Fig. 7*B*). COS cells were next transfected with either epitope-tagged wild-type or mutant IKKα and IKKβ, and following immunoprecipitation with the M2 monoclonal antibody these kinases were assayed using *in vitro* kinase assays with β-catenin and IκBα as substrates (48). Wild-type IKKα and IKKβ, but not the kinase-defective mutants, were able to phosphorylate β-catenin and IκBα (Fig. 7*C*).

Finally, we addressed whether IKKα and IKKβ could also phosphorylate additional regions in β-catenin other than its amino terminus (Fig. 7*D*). Both kinases phosphorylated GST fusion proteins containing various portions of β-catenin (Fig. 7*D*, *lanes 2–5*). These GST fusions contained either the amino terminus of β-catenin, an amino-terminal-deleted form of β-catenin or full-length β-catenin (Fig. 7*D*). Similar results were obtained using IKKα and IKKβ preparations produced by baculovirus expression (data not shown). These results indicate that both IKKα and IKKβ phosphorylate multiple regions of β-catenin.

Stoichiometry of IKK Phosphorylation of IκBα and β-Catenin—Next we compared the ability of IKKα and IKKβ to phosphorylate GST-IκBα-(1–54) and GST-β-cat-(1–91) substrates. In these *in vitro* kinase assays, we analyzed the phosphorylation of each of these substrates at specific points over a 120-min time course utilizing 0.01 mM, 0.1 mM, and 1.0 mM of cold ATP and 15 μCi of [γ-^{32}]ATP. Following SDS-PAGE and autoradiography (Figs. 8*A* and 8*B*, *top panels*), the ^{32}P-incorporation into the β-catenin and IκBα substrates was determined, and the number of the moles of phosphate incorporated per mole of

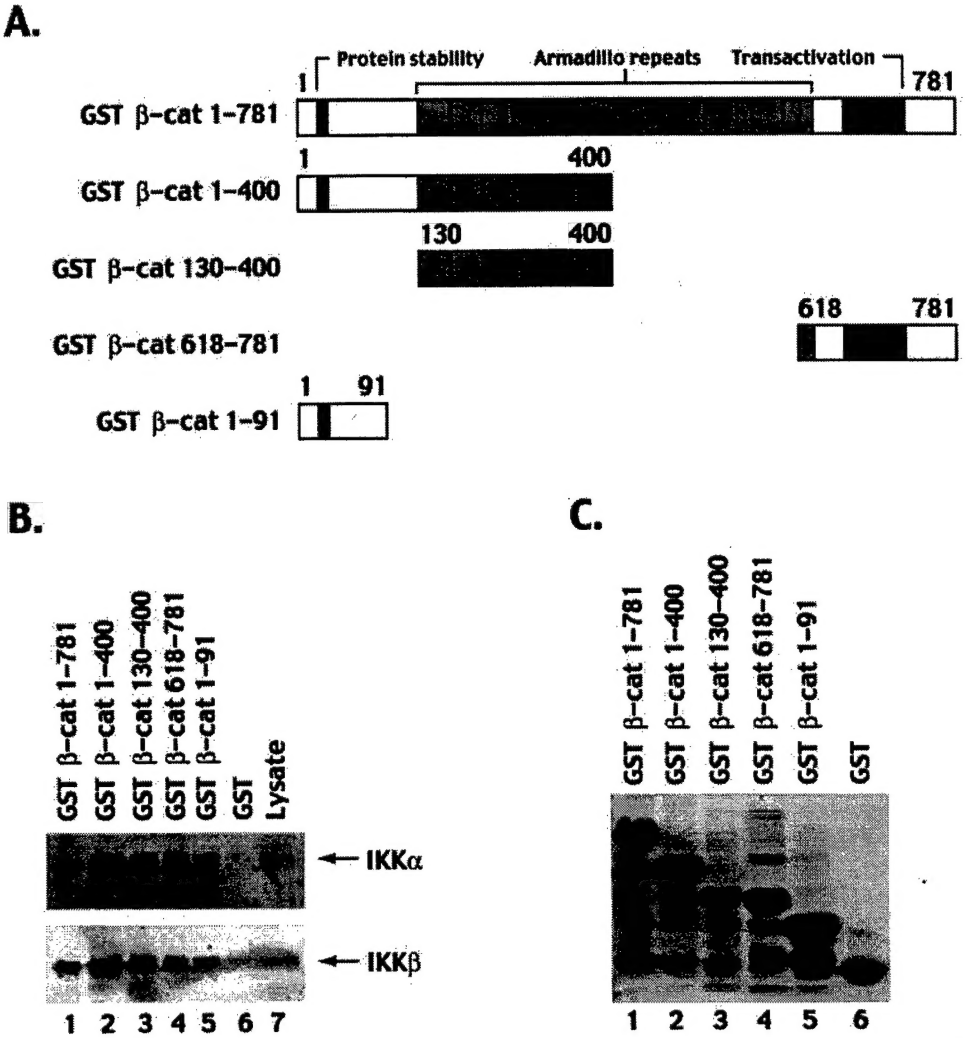

FIG. 6. **In vitro interaction of β-catenin with endogenous IKKα and IKKβ.** *A,* a schematic representation of the GST β-catenin fusion proteins that were used to analyze interactions with SW480 extract is shown. *B,* GST-fusion proteins with β-catenin were bound to glutathione-Sepharose beads (*lanes 1–6*), and after overnight incubation with SW480 cell extract, the glutathione beads were extensively washed and Western blot analysis was performed using rabbit polyclonal antibodies directed against either IKKα or IKKβ as indicated. 10% of the SW480 lysate alone is shown in *lane 7. C,* the GST-fusion proteins were analyzed by Coomassie Blue staining.

substrate was calculated (Fig. 8, *A* and *B*, *lower panels*).

Kinase assays performed with IKKα using 1 mM of cold ATP resulted in ~0.05 mol of phosphate/mol of protein incorporated into the amino terminus of β-catenin as compared with 0.09 mol of phosphate/mol of protein incorporated into the amino terminus of IκBα after a 120-min reaction (Fig. 8*A*). Kinase assays performed with IKKβ using 1 mM of cold ATP demonstrated that there was 1.4 mol of phosphate/mol of protein incorporated into the amino terminus of β-catenin and 0.5 mol of phosphate/mol of protein incorporated in the amino terminus of IκBα after 120 min (Fig. 8*B*). It is interesting to note that the phosphorylation of the β-catenin by IKKβ may be biphasic in contrast to its phosphorylation of IκBα (Fig. 8*B*). Similar phosphate incorporation into these substrates was found using both baculovirus-produced and COS-transfected IKKα and IKKβ proteins (data not shown). In agreement with previous studies, this analysis indicates that IKKα is a much weaker kinase than is IKKβ in phosphorylating IκBα (58) and β-catenin. These results indicate that the IKK proteins result in relatively similar incorporation of phosphate into the amino terminus of β-catenin and IκBα, although there are differences in the kinetics of this process.

DISCUSSION

In this study, we present data that IKKα and IKKβ can modulate β-catenin function. First, we observed the differential localization of β-catenin in mouse embryo fibroblasts derived from IKKα- and IKKβ-deficient cells. Second, the transcriptional activity of β-catenin was higher in IKKβ$^{-/-}$ cells as compared with IKKα$^{-/-}$ cells. Third, IKKβ decreased β-catenin-dependent gene expression similar to the effects seen with GSK-3β, while IKKα increased this activity. Fourth, we found that IKKα expression in COS cells increased the amount of β-catenin, while IKKβ expression reduced the amount of β-catenin. Finally, we demonstrated that IKKα and IKKβ interacted with and were able to phosphorylate β-catenin. Experiments are underway to map the sites in β-catenin that are phosphorylated by IKKα and IKKβ in order to determine whether phosphorylation alters β-catenin function. Our preliminary results suggest that IKKα phosphorylates different residues in the amino terminus of β-catenin than serine residues 33 and 37 that are phosphorylated by GSK-3β.

Studies with an amino-terminal deletion of β-catenin indicated that IKKα requires this region to increase β-catenin-de-

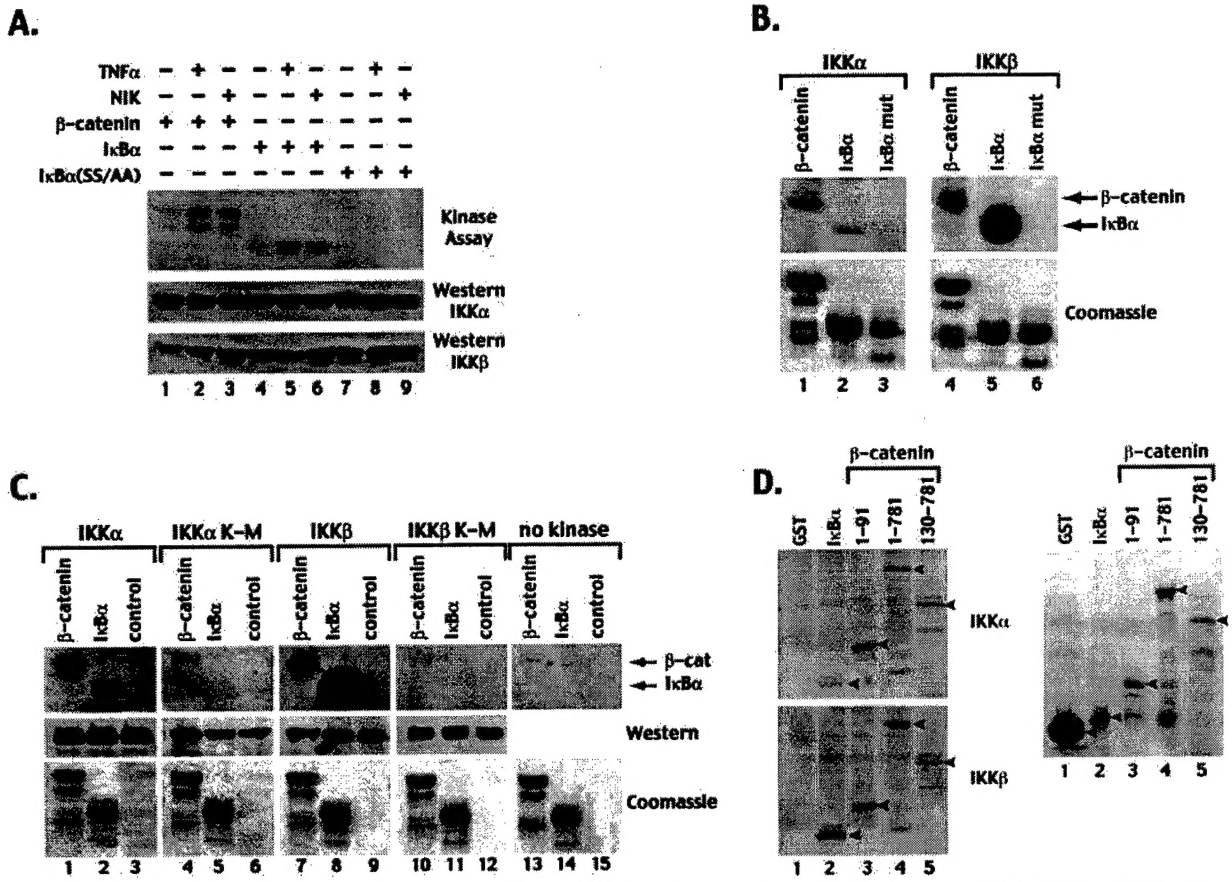

FIG. 7. **IKKα and IKKβ phosphorylate β-catenin and IκBα.** *A*, HeLa cells were either untreated, treated with TNFα (20 ng/ml) for 10 min, or transfected with a pCMV5 expression vector encoding NIK. Extracts were immunoprecipitated with a polyclonal antibody directed against IKKα and IKKβ and *in vitro* kinase assays were performed with GST fusions with β-catenin-(1–91), IκBα-(1–54) or IκBα SS/AA-(1–54) followed by autoradiography. *B*, recombinant baculovirus-expressed IKKα and IKKβ proteins were purified as described. *In vitro* kinase assays were performed using IKKα and IKKβ and GST-β-cat-(1–91) (*lanes 1* and *4*) and GST-IκBα-(1–54) (*lanes 2* and *5*) as substrates (*upper panel*). A GST-IκBα protein in which serine residues 32 and 36 were changed to alanine (*lanes 3* and *6*) was also assayed. The GST fusion proteins used in the *in vitro* kinase assay were monitored by Coomassie Blue staining (*lower panel*). *C*, COS cells were transfected with expression vectors encoding either FLAG-tagged wild-type or mutant IKKα or IKKβ kinases or mock transfected (*no kinase*). After 30 h, the cells were collected, and cellular extract was immunoprecipitated with the M2 monoclonal antibody directed against FLAG-epitope. The *upper panel* demonstrates *in vitro* phosphorylation of either GST-β-catenin (*lanes 1, 4, 7, 10,* and *13*), GST-IκBα (*lanes 2, 5, 8, 11,* and *14*) or no added substrate (*lanes 3, 6, 9, 12,* and *15*) by the indicated kinases. Expression of the transfected IKK constructs was analyzed by Western blot analysis (*middle panel*). Immunoprecipitation of extracts from mock-transfected cells were also analyzed in *in vitro* kinase assays (*lanes 13–15*). The amount of GST-β-catenin and GST-IκBα substrates used in these assays was monitored by Coomassie Blue staining (*lower panel*). *D*, FLAG-tagged IKKα (*top panel*) and IKKβ (*bottom panel*) immunoprecipitated from COS cell extracts were used in *in vitro* kinase assays with GST alone (*lane 1*) or GST fusions containing IκBα-(1–54) (*lane 2*), β-catenin-(1–91) (*lane 3*), β-catenin-(1–781) (*lane 4*), or β-catenin-(130–781) (*lane 5*). SDS-PAGE and autoradiography were then performed. A Coomassie-stained gel of the substrates used in the *in vitro* kinase assays is shown in the *right panel*.

pendent gene expression, while the effects of IKKβ on β-catenin activity are not dependent on this region. These results and the finding that IKKα is not able to increase the protein levels of an amino-terminal β-catenin mutant suggest that the amino terminus of β-catenin is likely involved in IKKα regulation. Thus, IKKα and IKKβ likely have effects on different domains of β-catenin to alter its role on gene expression. It is unclear whether differences in the kinase activity of IKKα and IKKβ are involved in their differential effects on β-catenin-dependent gene expression or whether other effects such as differential binding to specific pools of β-catenin may be involved. Finally, additional mechanisms such as IKK effects on β-catenin protein stability and/or nuclear import or export are possible. Multiple factors including Wnt signaling, the TCF/LEF proteins (19, 52, 53), and APC (59) affect the cellular localization of β-catenin, which lacks a canonical nuclear localization signal. Although we demonstrate that the IKK proteins interact with β-catenin, it is possible that IKK interaction with other components of the Wnt pathway such as APC may also be involved in regulating β-catenin function.

Both IKKα and IKKβ can form heterodimers and homodimers, and dimerization of these kinases is essential for their activity (33, 47, 60, 61). However, previous data has suggested that there is no synergy between IKKα and IKKβ in regulating their kinase activity (58). Given the wide disparity in their kinase activity, they may have other cellular targets in addition to IκB (58). The ability of these kinases to potentially associate with as yet unidentified cellular factors may alter their substrate specificity. Gene disruption studies indicate that IKKβ rather than IKKα is the critical kinase involved in the activation of the NF-κB pathway in response to treatment with either TNFα or IL-1β (37–39). The predominant cytoplasmic localization of IKKβ probably reflects the major role of this kinase in the phosphorylation of the IκB proteins that are localized in the cytoplasm bound to the RelA/p65 NF-κB protein (37–39). The results of our immunofluorescence studies

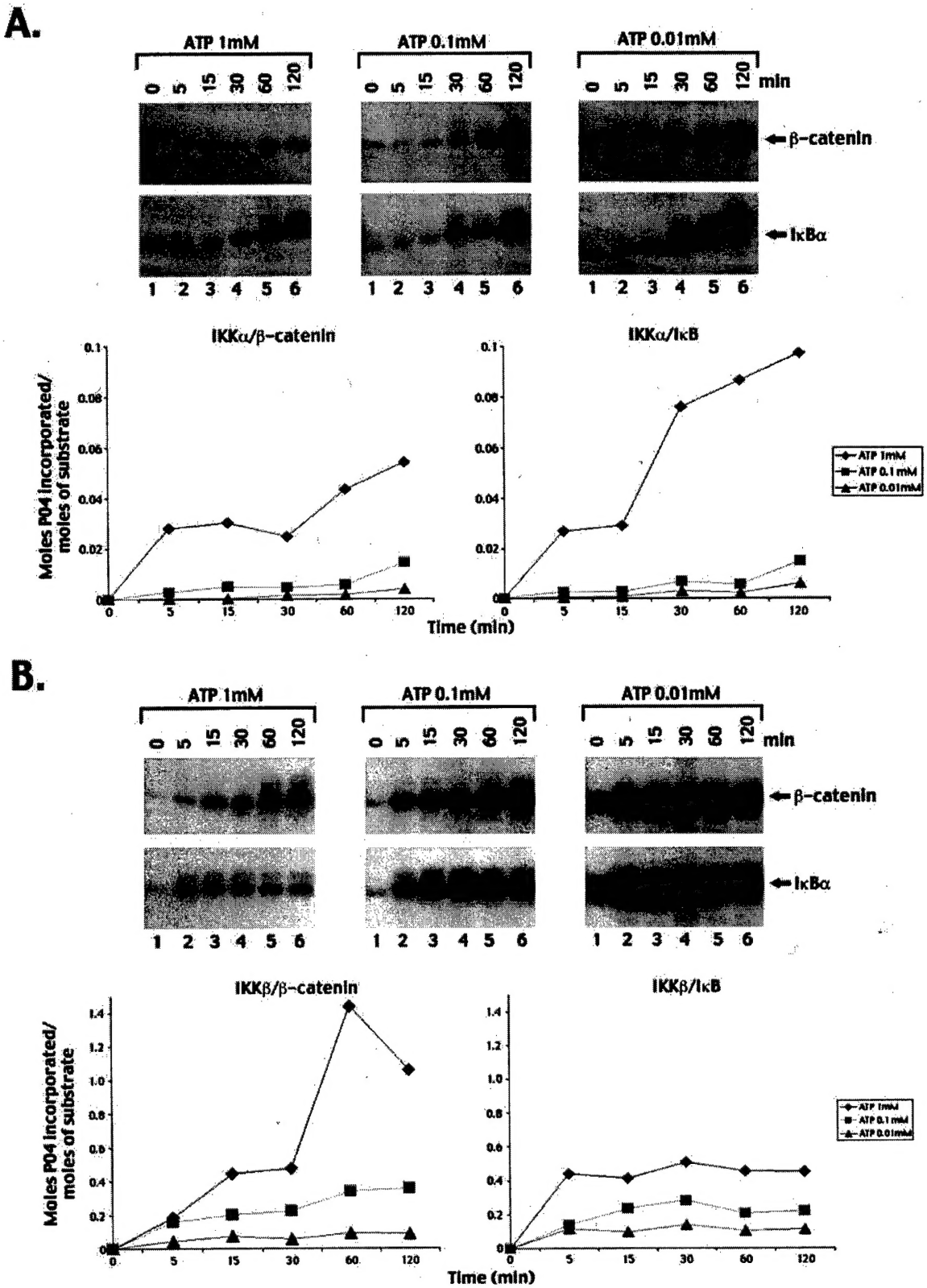

FIG. 8. **Stoichiometry of phosphate incorporation into the amino termini of IκBα and β-catenin by IKKα and IKKβ.** *A*, FLAG-tagged IKKα and *B*, IKKβ were immunoprecipitated from COS extracts with the M2 monoclonal antibody and incubated with 2 μg of GST fusions containing either β-catenin-(1–91) or IκBα-(1–54) in kinase buffer containing 15 μCi of [γ-^{32}P]ATP with a specific activity of 6000 Ci/mM and cold ATP at concentrations of 1 mM, 0.1 mM, and 0.01 mM. In vitro kinase reactions were performed for 0, 5, 15, 30, 60, and 120 min at 30 °C, and the samples were subjected to SDS-PAGE and autoradiography (*A* and *B*, *top panels*). Incorporation of ^{32}P into these substrates was quantitated by scintillation counting and the moles of phosphate incorporated per mole of substrate was calculated (*A* and *B*, *bottom panels*).

suggest that IKKα is localized in both the nucleus and cytoplasm of MEFs and may be predominantly nuclear in the absence of IKKβ in IKKβ$^{-/-}$ cells. Consistent with these observations, Western blot analysis of extracts prepared from COS cells transfected with expression vectors encoding IKKα and IKKβ indicate that IKKβ is predominantly localized in the cytoplasm, while IKKα is present in both the nucleus and the cytoplasm. Additional studies are currently underway to better characterize the cellular localization of IKKα. Whether any of the effects of IKKα on skin and skeletal development may in part be mediated by either IKKα binding and/or phosphorylation of β-catenin remains to be determined.

IKK regulation of β-catenin activity differs from its activation of the NF-κB pathway. Cytokines such as TNFα stimulate IKK phosphorylation of IκB leading to its rapid degradation and the nuclear translocation of NF-κB. TNFα activation of an NF-κB reporter construct is blocked by transfection of an IKKβ dominant negative mutant (30). Although TNFα treatment of cells results in marked decreases in β-catenin-dependent gene expression, this effect is only partially blocked by an IKKβ dominant negative mutant. These results suggest that the effects of TNFα on β-catenin-dependent gene expression likely involve additional substrates and/or pathways other than IKKβ and β-catenin. Although our results support a role for IKKα and IKKβ on modulating β-catenin activity, the regulation of this pathway is different from that seen with TNFα-induction of IKK to activate the NF-κB pathway.

Several observations are also consistent with the potential for similar factors being involved in the regulation of the Wnt and NF-κB pathways. It has been demonstrated that β-catenin/TCF signaling increases β-TrCP levels by a posttranscriptional mechanism to result in increased degradation of both β-catenin and IκB (45). Thus, changes of β-TrCP levels can result in marked effects on both the β-catenin and NF-κB pathways. Additionally, GSK-3β, which is an important kinase involved in regulating β-catenin levels, has also been implicated in regulating NF-κβ activation. Gene disruption studies have indicated that GSK-3β$^{-/-}$ mice have a phenotype similar to IKKβ-deficient mice developing liver degeneration as a result of increased sensitivity to TNFα stimulation (46). The mechanism by which GSK-3β may alter the NF-κB pathway remains to be determined. In summary, our studies suggest that a common set of cellular factors may be involved in the integration of a variety of cellular signaling processes that regulate the NF-κB and β-catenin pathways.

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